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13. ABSTRACT ***(Maximum 200 Words)***

These studies were based on the hypothesis that activated eosinophils release substances (mediators) that inhibit the growth of breast tumor cells in vitro. Cultured supernatants (24hr, 48hr and 72hr) from eosinophil cell lines were examined for growth inhibition of MCF-7 and MDA-MB-231 breast tumor cell colony formation. Serial 2-fold dilutions (1:2, 1:4 and 1:8) inhibited both MCF-7 and MDA-MB-231 colony formation in vitro. The time of harvesting of the supernatants affected their inhibitory capacity 72hr > 48hr > 24hr. Prior testing of eosinophil conditioned media revealed the presence of IL-4, and current examination revealed the presence of TNFα (>40pg/ml). When exogenous cytokines were used, TNFα potently inhibited colony formation of MCF-7, while IL-3, IL-4, IL-5 were marginal in their activity (10-35%) inhibitory activity. These data clearly illustrate that eosinophils produce mediators (some of which may be cytokines), which can inhibit tumor cell growth. Moreover, the data suggest potential collaborative activity of eosinophil mediators in the growth inhibition of tumor cells. The establishment of eosinophil cell lines offer a rich resource for the characterization of eosinophil mediators and further defining the role of eosinophils as anti-cancer effectors.

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Paulette Turbert-Harris

PI - Signature Date

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5. Introduction

Eosinophils are nonimmune inflammatory cells principally involved in type I hypersensitivity and parasitic infections (1). In addition to their granular proteins which are toxic to surrounding tissues during inflammatory reactions, eosinophils also produce an array of cytokines which in addition to autologously regulating them, may also have anti-cancer activities (2). Eosinophils also produce perforin and granzymes and hence have the capacity to kill tumor targets similarly to cytotoxic T lymphocytes and natural killer cells (3). In this investigation we have hypothesized that eosinophils, like NK cells may be non specific anti-cancer effector cells and that their mechanism of killing of tumor cells is two-tiered; l) by binding to and releasing toxic granules like perforin and granzymes which cause the tumor cells to die by apoptosis and 2) by release of cytokines which inhibit tumor growth. We have immortalized subpopulations of eosinophils from individuals with mild to moderate eosinophilia. Hypo- and hyperdense eosinophils were collected from metrizamide density gradient fractions 22 and 24. These cells have been characterized by flow cytometry using a panel of fluorescein-labelled antibodies to surface markers found on eosinophils. Previous reports demonstrated that these cell lines like fresh peripheral blood eosinophils inhibit tumor cell growth. In this report we demonstrate the ability of conditioned media (cultured supernatants) and exogenous cytokines to inhibit tumor cell colony formation.

6. Body

Collection of Cultured Supernatants from Eosinophil Cell Lines. Eosinophil cell lines (4) and sublines (3) were incubated at $1x10^6$ /ml for 24, 48 and 78hrs in RPMI medium supplemented with sodium pyruvate (l mM), non-essential amino acids (l mM), penicillin/streptomycin (50 units/ml, respectively), gentamycin (50 ug/ml) complete medium and 1% fetal bovine serum. The supernatants were collected after centrifugation at 2,000rpm for 30 mins, aliquoted and stored at –20°C.

Growth Inhibition of MCF-7 and MDA-MB-231 tumor cells by Eosinophil Cultured Supernatants.

a) Monolayer- MCF-7 and MDA-MB-231 tumor cells were seeded into the wells of a 6-well cluster plate at $lx10^5$ cells per well, and incubated overnight (16-24hr) at 37°C, 5% CO_2. Supernatants from eosinophil cultures were added (l ml/well). Fresh complete RPMI medium containing 10% fetal bovine serum was added to triplicate wells per 6-well cluster. The plates were further incubated for an additional 48hrs or until the control wells were confluent. The eosinophils were removed from the wells which were washed 3x with phosphate buffered saline, then stained with hematoxylin and eosin. Photo microscopic documentation was carried out. Cell line GRC.014.24.S1 was unable to inhibit the growth of MRC-5 fibroblasts at 1:1, 2:1 and 5:1 E:T ratios (fig 2). On the otherhand, at 2:1 and 5:1, MCF-7 cells were inhibited. Cell line (fig 3) GRC.014.22S completely (fig 3) abrogated MCF-7 growth at 1:1 and 2:1 E:T ratios. Growth inhibition of MDA-MB-231 by these cell lines was more varied. The greatest inhibition with both cell lines was at the E:T ratio of 2:1.

b)Colony formation - Tumor cells were seeded into 6-well plates at 100 cells per well. The plates were incubated overnight at 37°C. Conditioned media supernatants from eosinophil cell line cultures (24, 48 and 72hrs) were added to the wells in total volume of 2mls. Serial 2-fold dilutions of the conditioned supernatants were made and each dilution was added in triplicate to the cells. Each plate contained triplicate control wells (10% RPMI medium). The plates were then incubated for 10 days at 37°C, 5% CO_2. The wells were washed carefully with phosphate buffered saline, then stained with hematoxylin and eosin. Manual counts were taken and percent inhibition determined.
Concentrated undiluted supernatants completely inhibited colony formation. With the 1:2 dilution of cultured supernatant from the hypodense cell line GRC.014.22 (fig 5), there was 73% inhibition of MCF-7 colony formation and 93% with both the 48hr and 72hr supernatants. At the 1:4 dilution there was 69% inhibition with the 24hr supernatant, 81% with the 48hr supernatant and 88% with the 72hr preparation. At the 1:8 dilution 30% inhibition occurred with the 24hr supernatant, 52% with the 48hr supernatant and 71% with the 72hr preparation.
When MDA-MB-231 tumor cells were analyzed, both the concentrated and the 1:2 dilution inhibited colony formation by 100% (fig 6). However, with the 1:4 and 1:8 dilutions, 24hr, 48hr and 72hr were quite similar in pattern in that 30-45% inhibition was obtained. When the 1:2 diluted supernatants from the eosinophil cell line GRC.014.24 (fig7) were assayed, there was an increase in inhibition (81% to 93%) with the 24hr and 48hr supernatants. This decreased slightly to 87% with the 72hr sample. With the 1:4 dilution, there was 65% inhibition with the 24hr supernatant, 91% and 92% with the 48hr and 72hr preparations. With the 1:8 dilutions, MCF-7 colony growth was inhibited by 34% with the 24hr supernatant, 72% and 73% with the 48hr and 72hr preparations. MDA-MB-231 tumor cells (fig 8) were more resistant than MCF-7 cells to the 1:4 and 1:8 dilutions from all 3 culture times (24hr, 48hr and 72hr). The percent inhibition of colony formation for the 1:4 dilutions of the three culture preparations were 37, 50 and 60%, respectively and very similar for the 1:8 at 37, 40 and 60%, respectively. The 1:2 dilution of all three preparations inhibited colony formation by 100%.

A similar pattern of activity though lower was observed with cell line SD.031.22 (fig 9) in that when the 24hr supernatant was tested on MCF-7 cells, at 1:2 dilution there was 60% inhibition, 55% at 1:4 and 25% at 1:8. The pattern of activity was the same for both the 48hr and 72hr cultured supernatants however the percent inhibition was higher, 74%, 70%, 38% (48hr); and 97%, 74% an 38% (72hr). Activity against MDA-MB-231 was very similar (fig 10). Cultured Supernatants from eosinophil cell line SD.031.24 inhibited MCF-7 (fig 11) tumor cells similarly to the previous cell lines GRC.014.22 (fig 5) and GRC.014.24 (fig 7) and SD.031.22 (fig 9). Inhibition by the 24hr supernatants was 92%, 60% and 54% for the 1:2, 1:4 and 1:8 dilutions, respectively. Inhibition by the 48hr supernatant was 98%, 88% and 75% for the same dilutions, respectively. The 72hr cultured supernatant showed the same pattern of inhibition. The inhibitory activity of SD.031.24 supernatants against MDA-MB-231 (fig 12) was less than that against MCF-7, similarly to the GRC.014.24 supernatants. Sublines of the GRC.014 cell lines were developed by sterile sorting with a Fluorescent Activated Cell Sorter using CCR3 (Eotaxin Receptor) and CD49d as markers. Supernatants (24hr, 48hr and 72hr) from FACS sorted eotaxin receptor positive GRC.014.22 cells (referred to as GRC.014.22.S) (Fig 13) inhibited MCF-7 colony formation by 100% , 88% and 71% (1:2, 1:4 and 1:8, respectively), 100%, 91% and 64% (48hr); 100%, 84% and 77% (72hr). At 1:4 and 1:8 dilutions (fig 14) MDA-MB-231 cells were inhibited by ≥50%. Against MCF-7, the GRC.014.24S eotaxin receptor positive eosinophil

supernatants markedly inhibited colony formation 90%, 92% and 96% from 1:2 dilutions of 24hr, 48hr and 72hr cultured supernatants (fig 15). At 1:4 dilutions, inhibition was 50%, 75% and 85% for 24, 48 and 72hr cultures, respectively and at the 1:8 dilution percent inhibition was 26%, 54% and 63% for 24, 48 and 72hr cultured preparations, respectively. These supernatants also inhibited MDA-MB-231 cells (fig 16).
The last cell line tested was established from the GRC.014.24S subline, using the FACS Sorter and second eosinophil marker, CD49d. These cells are $CCR3^{+}$. Cultured supernatants from these cell line also inhibited MCF-7 (fig 17) and MDA-MB-231 (fig 18) in a dose-dependent manner.

Cytokine Presence in Eosinophil Cultured Supernatants.
24hr, 48hr and 72hr cultured supernatants from 7 eosinophil cell lines (parent and sublines) were evaluated by enzyme-linked immunoassay (ELISA) analysis for the presence of TNFα. TNFα was found in all samples at >40 pg/ml concentration.

Effect of Exogenous Cytokines on MCF-7 Colony Formation
IL-3 (fig 19) at 10ng/ml inhibited MCF-7 colony formation by 15%; at 50ng/ml by 30%, 0% at 100ng/ml and 26% at 200ng/ml. IL-4 inhibited colony formation by 10% at 10ng/ml, 25% at 50ng/ml and 14 at 100ng/ml. IL-5 at 10ng/ml failed to inhibit colony formation at 50 and 100ng/ml exerted 20% inhibition. At 10ng/ml IL-3 inhibited MDA-MB-231 (fig 20) colony formation by 10%, but had little to no effect any of the higher concentrations. At 10ng/ml, IL-4 inhibited colony formation by 17%, 28% at 50ng/ml, 20% at 100ng/ml and 16% at 200ng/ml. At 100ng/ml IL-5 inhibited tumor growth by 36%. TNFα on the otherhand, dramatically inhibited colony inhibition of MCF-7 cells (fig 21)

7. Key Research Accomplishments

- Promotion to Associate Professor
- Development of eosinophil sublines from 2 parental lines (3) based on FAC Sorting, using eosinophil markers
- Established inhibition of tumor growth by all 4 parent lines as well as 3 sublines

8. Reportable Outcomes

- Activated eosinophil subpopulations kill breast tumor cells in vitro. Furbert-Harris PM, Howland C, Hunter KA, Laniyan I, Vaughn T, Dunston GM, Parish-Gause D, Harris D, Abdelnaby A, Anderson D, Brown R, Awich J, Sumner s and Oredipe O. Era of Hope Department of Defense Breast Cancer Research Program, Proceedings Vol. II p. 674.
- Stimulation by swainsonine of myocardial sulfhydryl levels in high-dose doxorubicin chemotherapy. OA Oredipe, I Laniyan, WM Griffin, D Parish-Gause, T Vaughn, WR Green and PM Furbert-Harris. FASEB 14(8) 1155.

9. Discussion/Conclusion

We hypothesized that activated esoinophils and eosinophil cell lines inhibit breast cancer cell growth by releasing inflammatory substances that slow down the growth of the cells or are toxic to the cells, thereby causing their death. In this phase we have analyzed seven eosinophil cell lines, parent and sublines which we have developed by EBV-immortalization of metrizamide density gradient fractions of hypo-(M22) and hyperdense (M24) eosinophils. These cells were obtained from the peripheral blood of individuals (prior study) with mild to moderate eosinophilia. We have collected cultured supernatants from these cell lines, (which have been determined to inhibit the growth of breast tumor cells in vitro), and analyzed them for their inhibitory action on MCF-7 and MDA-MB-231 tumor cells. Cultured supernatants (24hr, 48hr and 72hr) from all 7 cell lines dramatically inhibited colony formation of both MCF-7 and MDA-MB-231 tumor cells. Undiluted, preparations of the cultured supernatants completely abrogated the colony growth. Serial two-fold dilutions, 1:2, 1:4 and 1:8 inhibited tumor colony formation in a dose-dependent manner. Supernatant preparations from 24hr, 48hr and 72hr cultures demonstrated inhibition dose-dependently. All supernatants were examined for TNFα and all contained >40 pg/ml. Exogenous cytokines which are known to regulate eosinophil differentiation and activation (I L-3, IL-5, GM-CSF) and IL-4 and TNFα, all of which can be produced by activated eosinophils, were examined for tumor cell growth inhibitory activity. There was marginal growth inhibition with all except TNFα. IL-3 at 50 ng/ml inhibited MCF-7 colony formation by 30% while IL-5 at 100 ng/ml inhibited MDA-MB-231 colony formation by 36%. The data were variable with regards to dose with IL-3, IL-4, IL-5 and GM-CSF. Overall, inhibition ranged from 0-35%. TNFα was markedly potent in its inhibitory activity.

These data confirm our hypothesis that mediators released by eosinophil cell lines inhibit tumor cell growth. Further analysis of eosinophil supernatants will be performed in order to better characterize the mediator participants active in eosinophil anti-cancer cytotoxic activity. These cell lines offer an excellent resource for studying the biology and functional activity of eosinophils.

10. References

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2. Molet S, Gosset P, Vanhee D, Tillie-Leblond I, Wallaert B, Capron Ma and Tonnel AB. Modulation of cell adhesion molecules on human endothelial cells by eosinophil-derived mediators. Jl Leuk Biol 63:35l-58, 1998.
3. Kuby J. Immunology (4^{th} ed) RA Goldsby, Thomas J Kinat, Barbara A Osborne (eds). WH Freeman & CO., 2000.

11. Appendices

Fig.1 Cytospin preparation of Eosinophil Cell Line stained with H&E

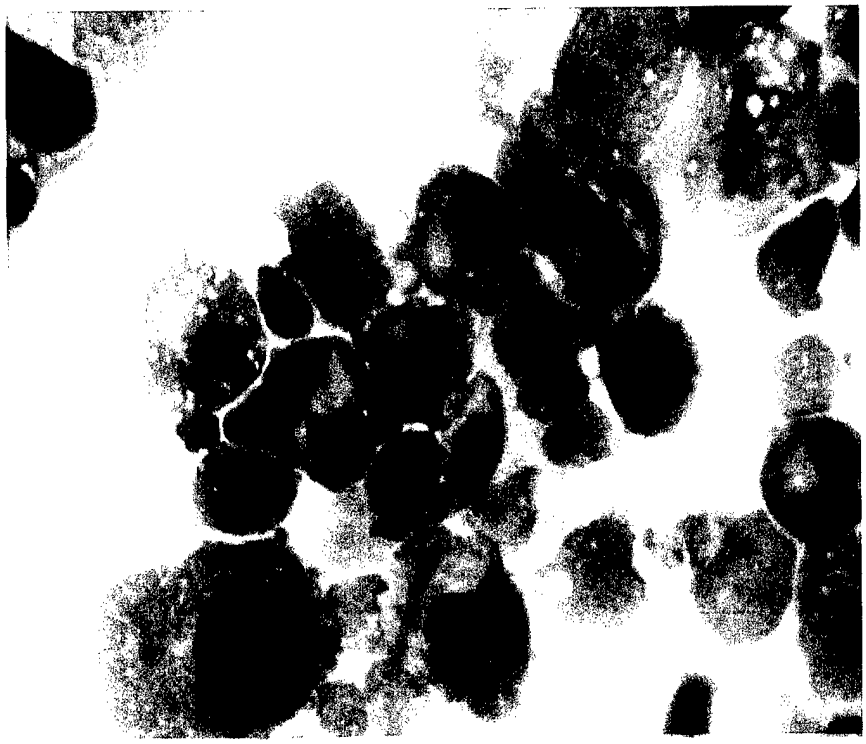
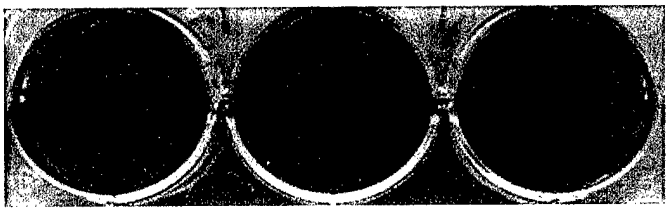
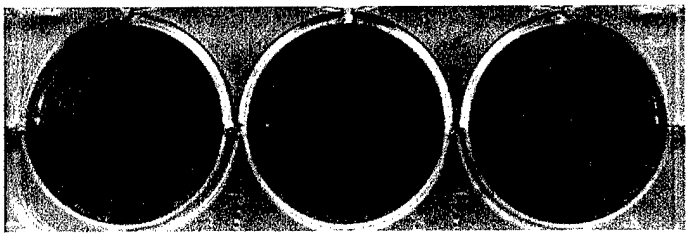


Fig 1. Cytospin preparation of eosinophil cell line stained with H & E

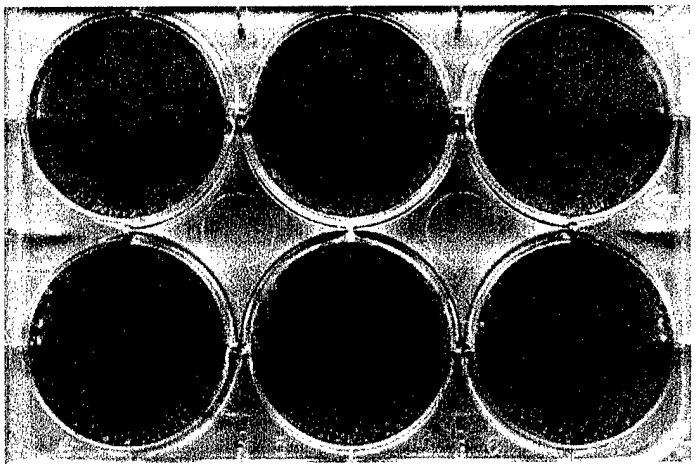
Fig.2 Eosinophil Cytotoxic Activity on MRC-5 Fibroblast Cell Growth



Control - MRC-5



GRC.014.24.S1 (1:1)

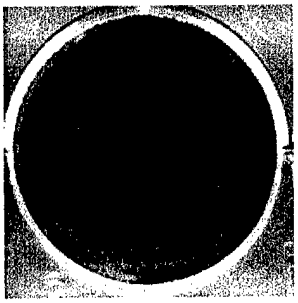


GRC.014.24.S1 (2:1)

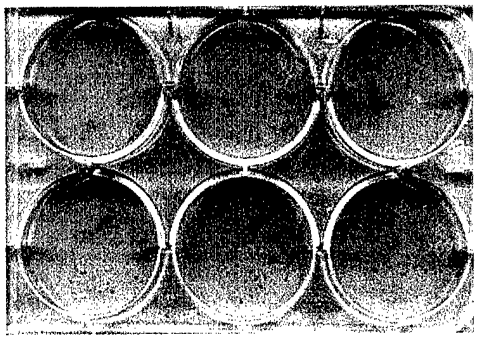
GRC.014.24.S1 (5:1)

Fig. 2. MRC-5 human lung fibroblasts were seeded into the wells of a 6-well plate at l.5 x 10^5 cells/well and incubated overnight at 37°C. The eosinophil subline GRC.014.24 Sl was then added to the monolayers at effector to target (E:T) ratios of l:1, 2:1, and 5:l. RPMI complete medium containing 10% fetal bovine serum was added to control wells. The experiments were performed in triplicate. The plates were further incubated for 48hrs or until the control wells were confluent.

Fig.3 Eosinophil Cytotoxic Activity on MCF-7 Cell Growth

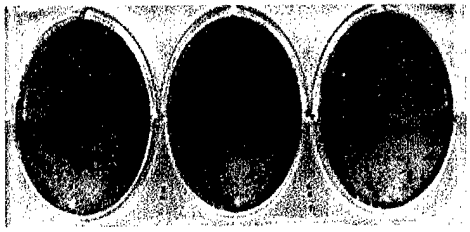


Control - MCF-7



GRC.014.22.S (1:1)

GRC.014.22.S (2:1)



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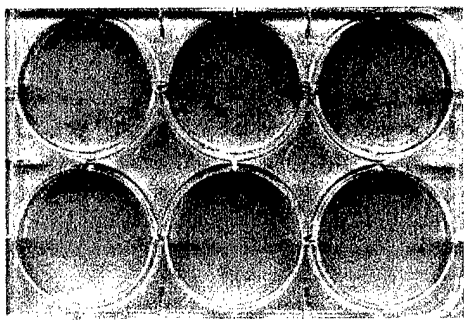
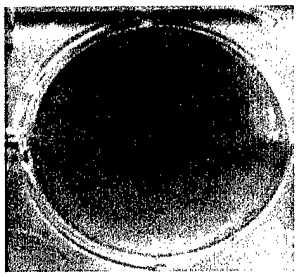


GRC.014.24.S1 (2:1)

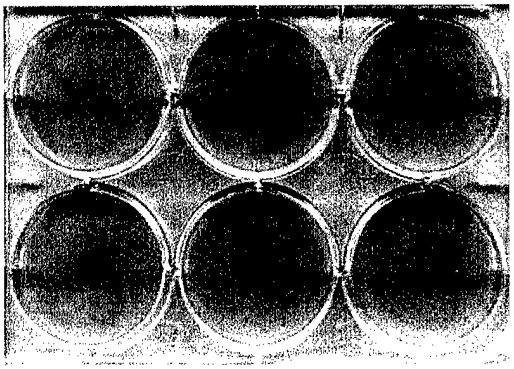
GRC.014.24.S1 (5:1)

Fig. 3. MCF-7 tumor cells were seeded into the wells of 6-well cluster culture plates at 1.5 x 10^5 cells/well and incubated overnight. Eosinophil cell lines GRC.014.22S and GRC.014.24Sl at E:T ratios of l:l, 2:l and 5:l. The plates were incubated for an additional 48hr or until the control wells were confluent.

Fig.4 Eosinophil Cytotoxic Activity on MDA MB 231 Cell Gowth

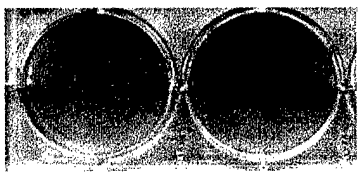


Control - MDA MB 231



GRC.014.22.S (1:1)

GRC.014.22.S (2:1)



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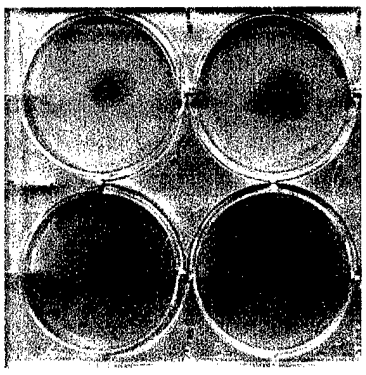

GRC.014.24.S1 (2:1)

GRC.014.24.S1 (5:1)

Fig. 4. MDA tumor cells were seeded into the wells of 6-well cluster culture plates at l.5 x 10^5 cells/well and incubated overnight. Eosinophil cell lines GRC.014.22S and GRC.014.24Sl at E:T ratios of l:l, 2:l and 5:l. The plates were incubated for an additional 48hr or until the control wells were confluent.

Fig.5 GRC.014.22 Eosinophil Supernatants Inhibit MCF-7 Colony Formation In Vitro

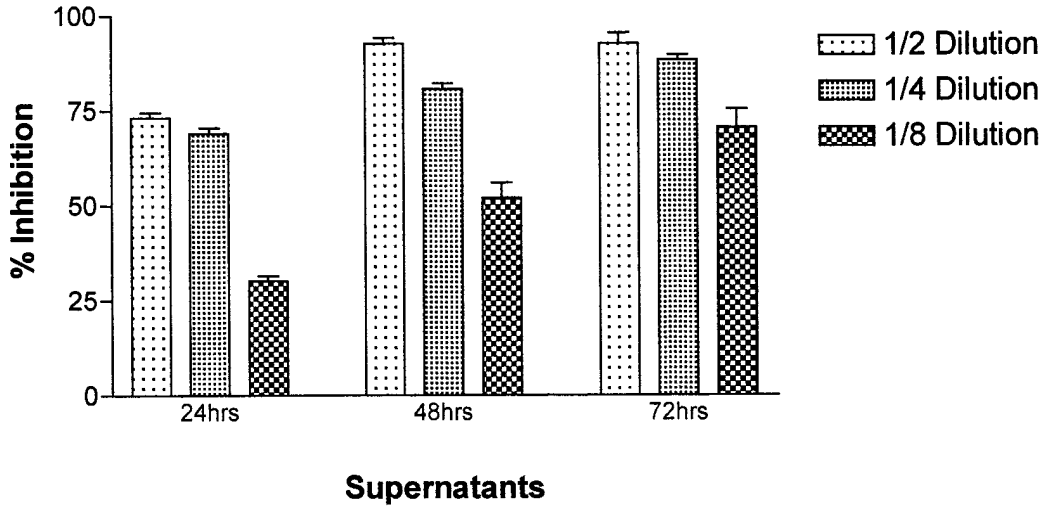

Fig. 5. MCF-7 tumor cells were seeded into wells of a 6-well plate at 100 cells per well and incubated overnight. The cells were then incubated with cell free supernatants from eosinophil cell line GRC.014.22 cultures for 10 days at 37°C. Plates were washed 3x with phosphate-buffered saline; stained with H & E, then counted.

Fig.6 GRC.014.22 Eosinophil Supernatants Inhibit MDA MB 231 Colony Formation In Vitro

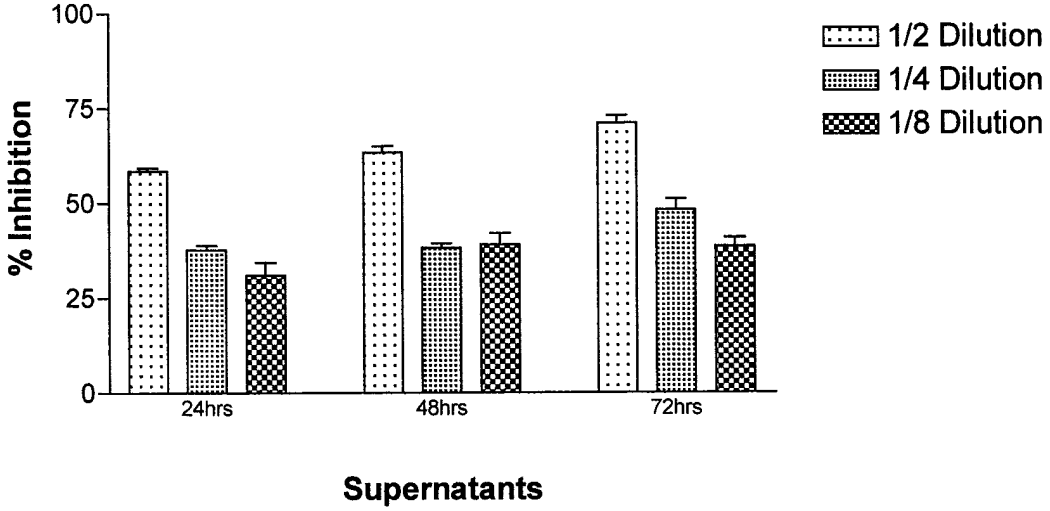

Fig. 6. MDA-MB-231 tumor cells were treated GRC.014.22 supernatants similarly to that described for MCF-7 in fig. 5. The colonies were stained and counted. Percent inhibition was determined.

Fig.7 The Effect of GRC.014.24 Eosinophil Cultured Supernatants on MCF-7 Breast Tumor Cells

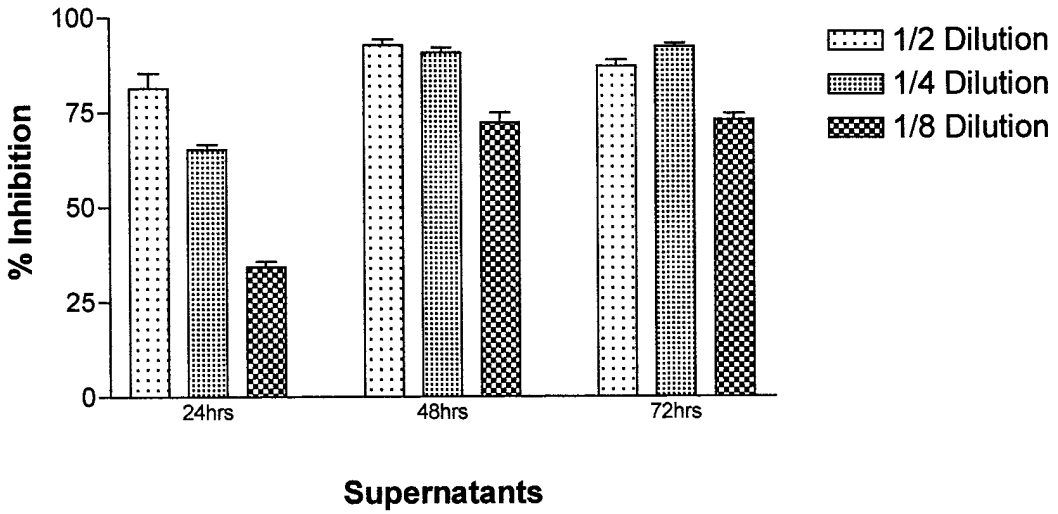

Fig.8 The Effect of GRC.014.24 Eosinophil Cultured Supernatants on MDA MB 231 Colony Formation

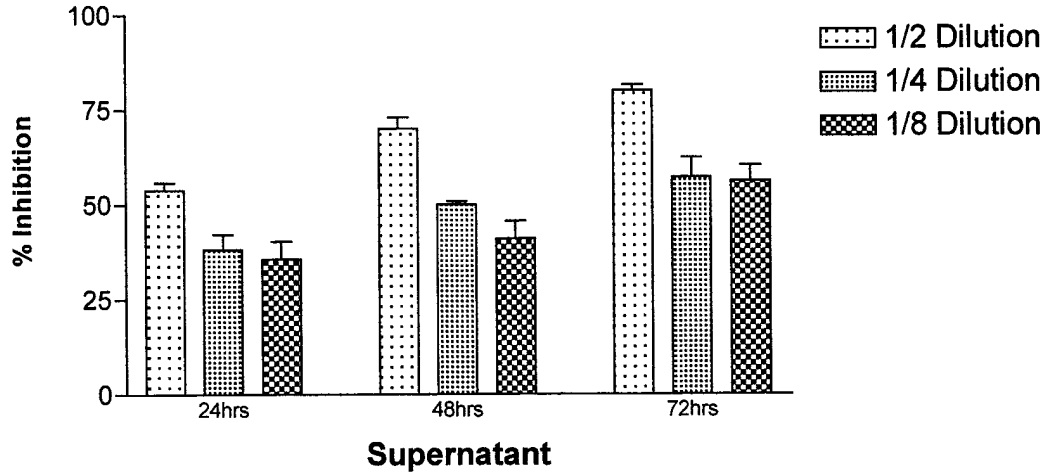

Fig. 7, 8. Cultured supernatants from GRC.014.24 eosinophils were examined for inhibitory activity against MCF-7 and MDA-MB-231 colony formation.

Fig.9 Effect of SD.031.22 Eosinophil Cultured Supernatants on MCF-7 Colony Formation

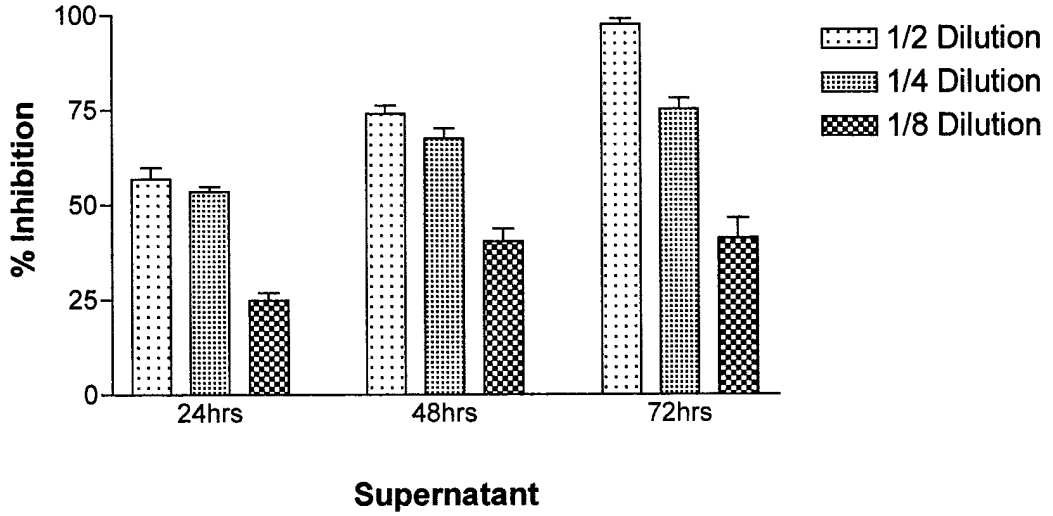

Fig.10 Effect of SD.031.22 Eosinophil Cultured Supernatants on MDA MB 231 Colony Formation

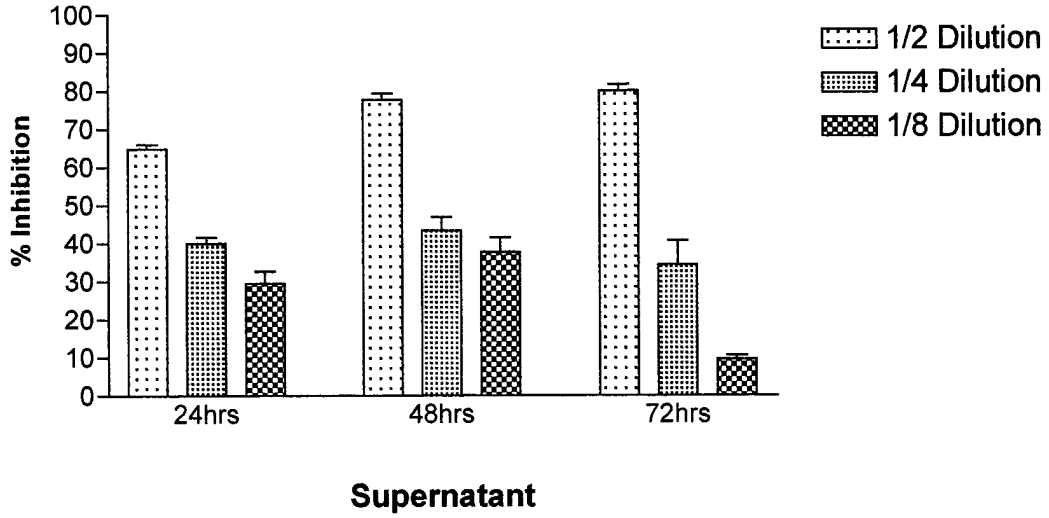

Fig. 9, 10. Cultured supernatants from eosinophil cell line SD.031.22 were incubated with MCF-7 and MDA-MB-231 tumor cells. The cells were incubated for 10 days as described previously. After harvesting, and staining, the colonies were counted and the % inhibition determined.

Fig.11 Effect of SD.031.24 Eosinophil Cultured Supernatants on MCF-7 Colony Formation

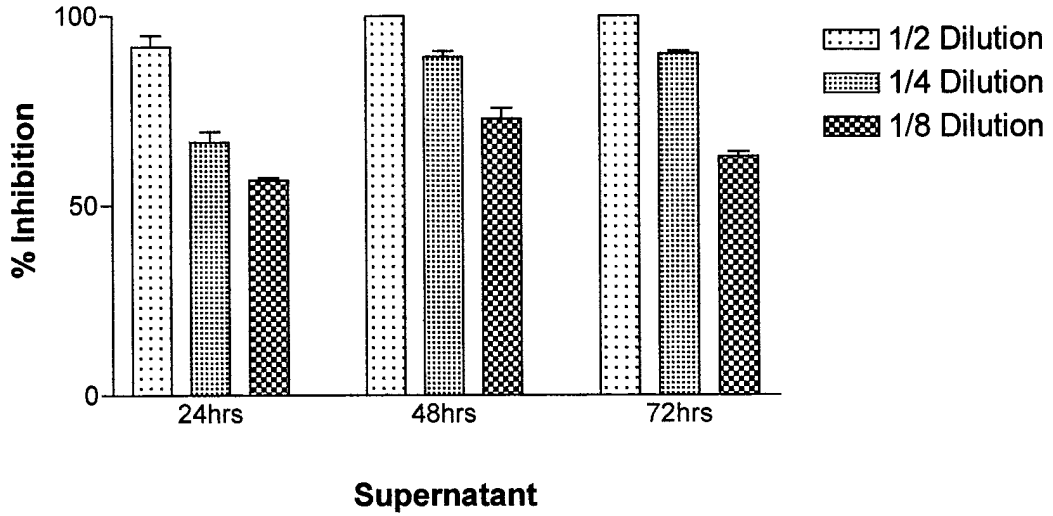

Fig.12 Effect of SD.031.24 Eosinophil Cultured Supernatants on MDA MB 231 Colony Formation

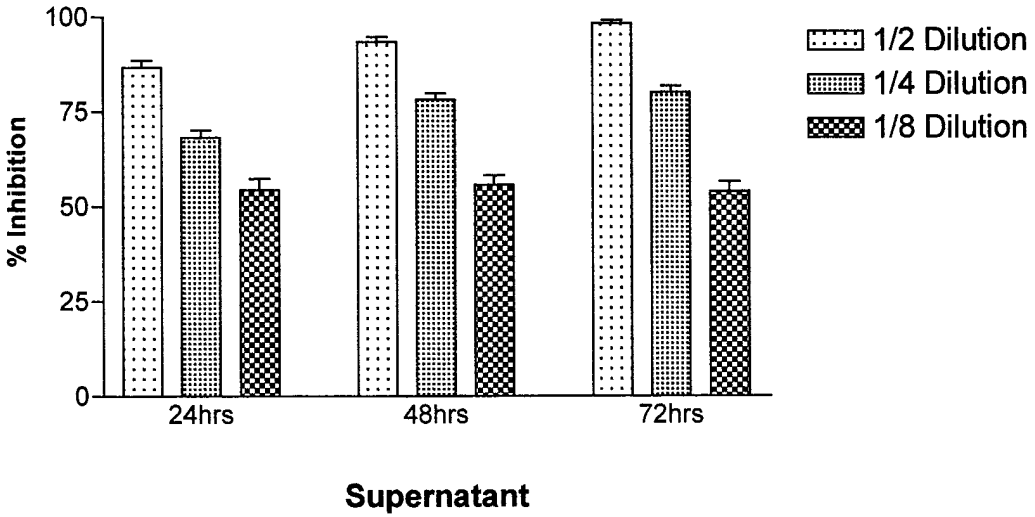

Fig.11, 12. MCF-7 (Fig 11) and MDA-MB-231 tumor cells were seeded into 6-well plates (l00 cells/well) and incubated overnight. Control wells contained RPMI complete medium with 10% FBS. Serial toe-fold dilutions of cultured supernatants from the eosinophil cell line SD.031.24 were added and the plates were incubated for an additional l0 days. Colonies were stained with H & E and counted. Percent inhibition was determined.

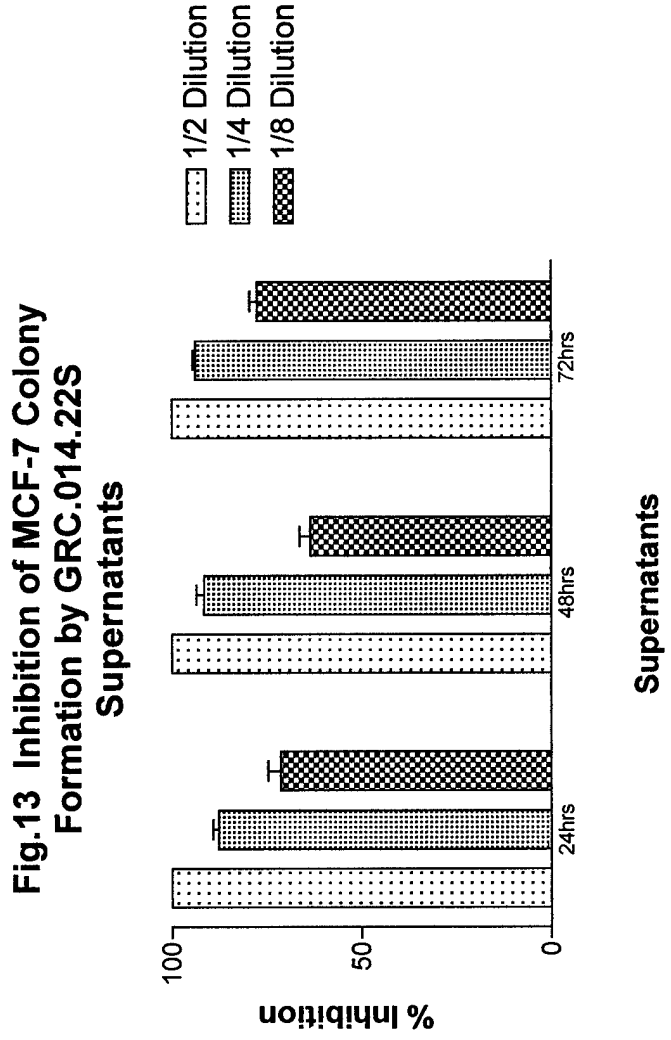
Fig.13 Inhibition of MCF-7 Colony Formation by GRC.014.22S Supernatants
1/2 Dilution
1/4 Dilution
1/8 Dilution
% Inhibition
100
50
0
24hrs
48hrs
72hrs
Supernatants

Fig.14 Inhibition of MDA MB 231 Colony Formation by GRC.014.22S Supernatants

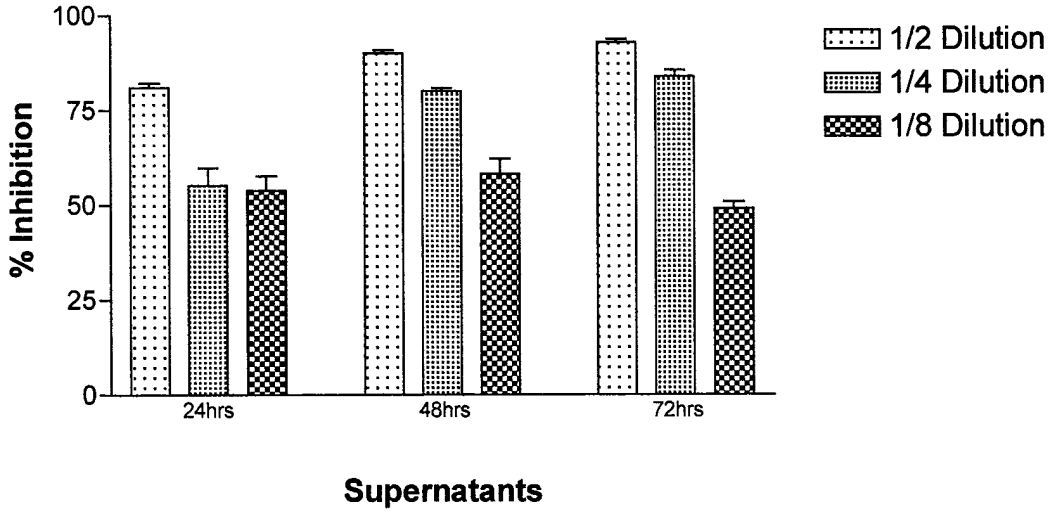

Fig. 13, 14. Supernatants from the eotaxin receptor positive subline GRC.014.22S were incubated with tumor cells MCF-7 and MDA-MB-231 (100 cell/well) in 6-well plates as described previously. Percent inhibition of colony formation was determined.

Fig.15 Cultured Supernatants from an Eotaxin Positive (CCR3+) Eosinophil Cell Line (GRC.014.24S) Inhibit MCF-7 Colony Formation

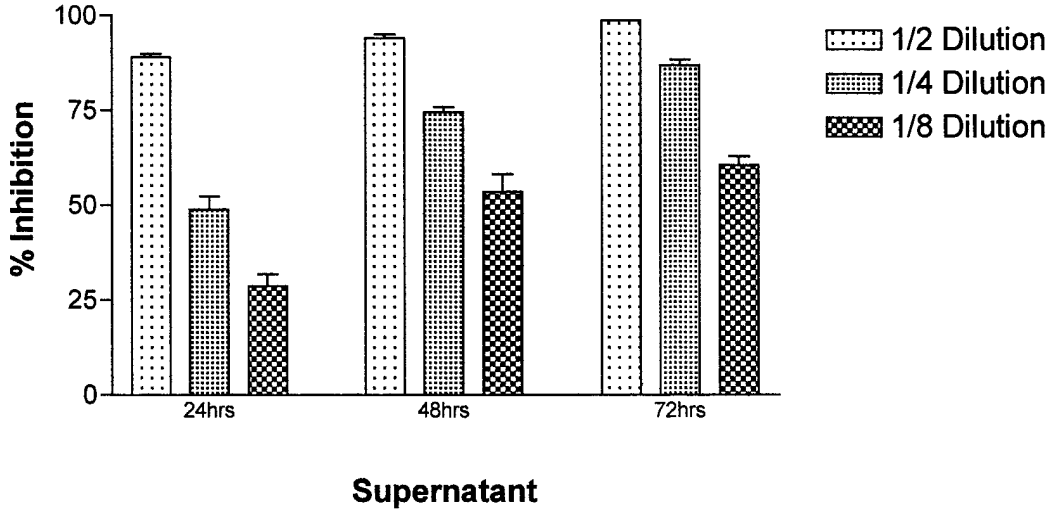

Fig.16 Cultured Supernatants from an Eotaxin Positive (CCR3+) Eosinophil Cell Line (GRC.014.24S) Inhibit MDA MB 231 Colony Formation

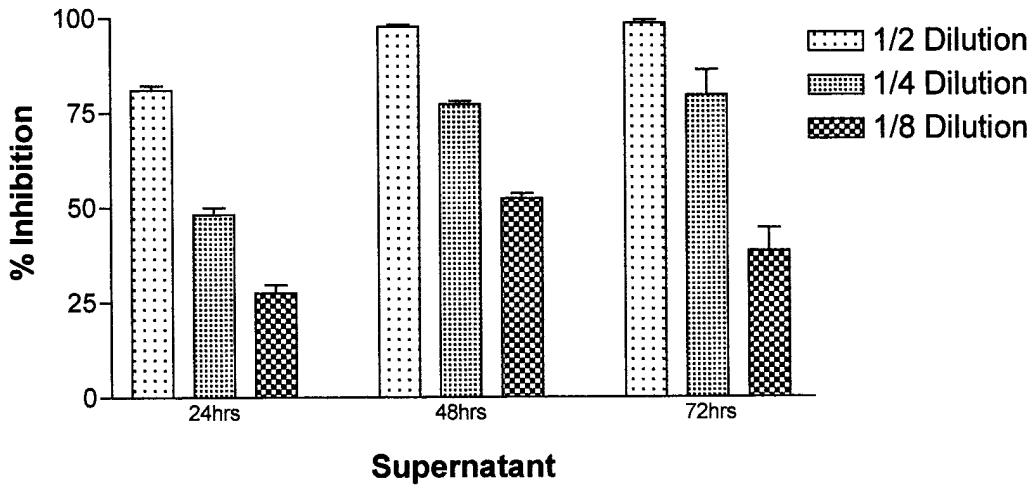

Fig. 15, 16. Cultured supernatants from the CCR3$^+$ eosinophil line GRC.014.24S similarly to that described in figures 13 and 14.

Fig.17 Cultured Supernatants from CCR3+, CD49d+ Eosinophil Cell Line Inhibit MCF-7 Colony Formation

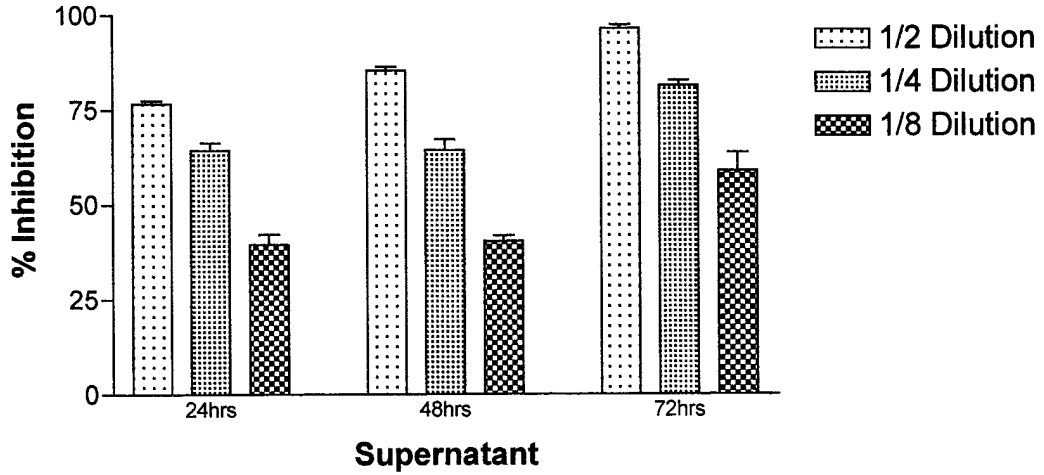

Fig.18 Cultured Supernatants from a CCR3+, CD49d+ Eosinophil Cell Line Inhibit MDA MB 231 Colony Formation

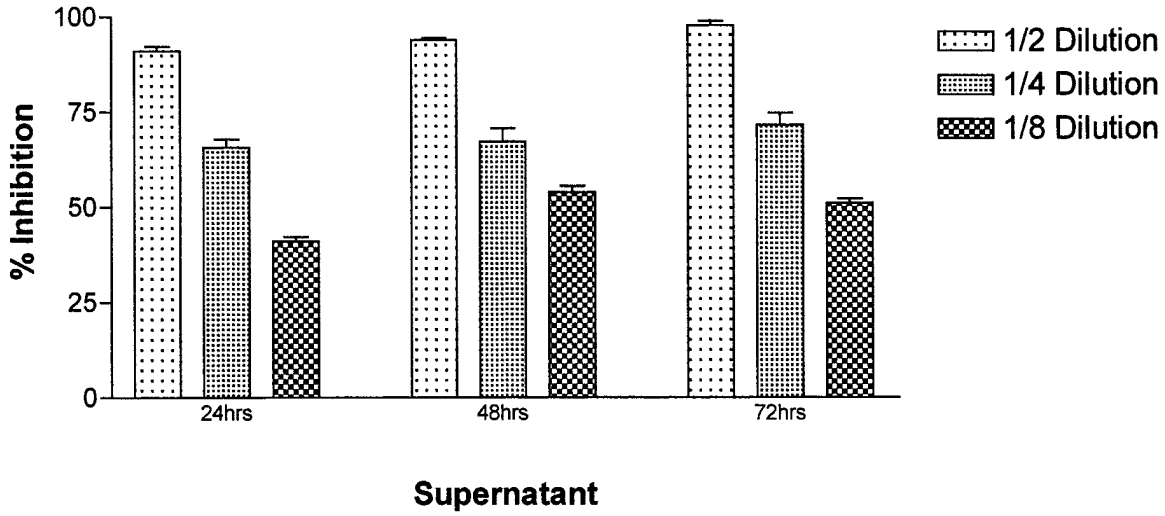

Fig. 17, 18. Cultured supernatants from $CCR3^+$ cells that were sorted using the CD49d marker, were incubated with MCF-7 and MDA-MB-231 tumor cells similarly to that described in figure 13 and 14.

Fig.19 The Effect of Cytokines on MCF-7 Colony Formation

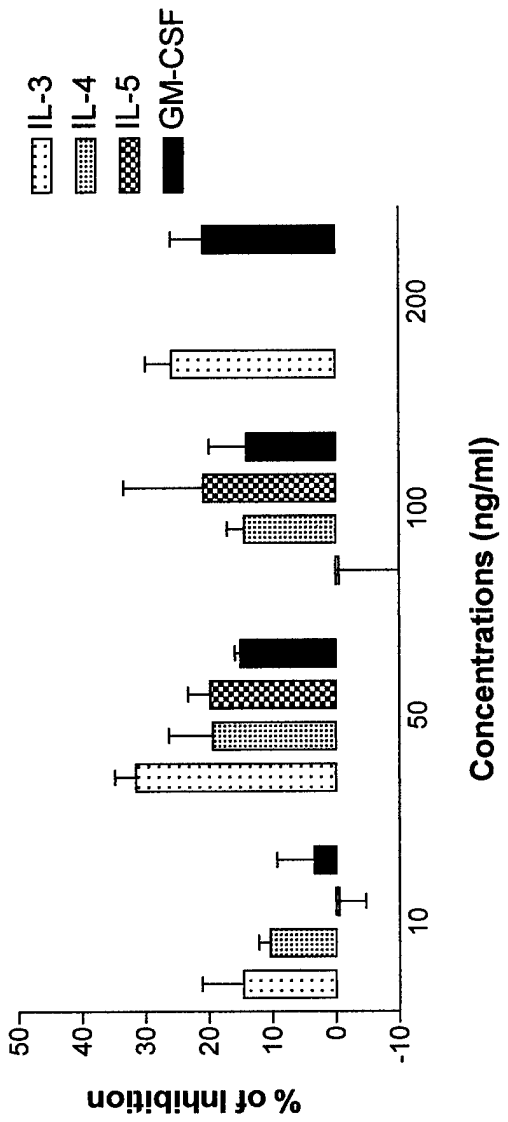

Fig. 19. MCF-7 cells were seeded into 6-well plates (100 cell/well), then treated with cytokines IL-3, IL-4, IL-5, GM-CSF at 10, 50, 100 and 200ng/ml. The plates were incubated for 10 days; harvested; colonies stained then counted.

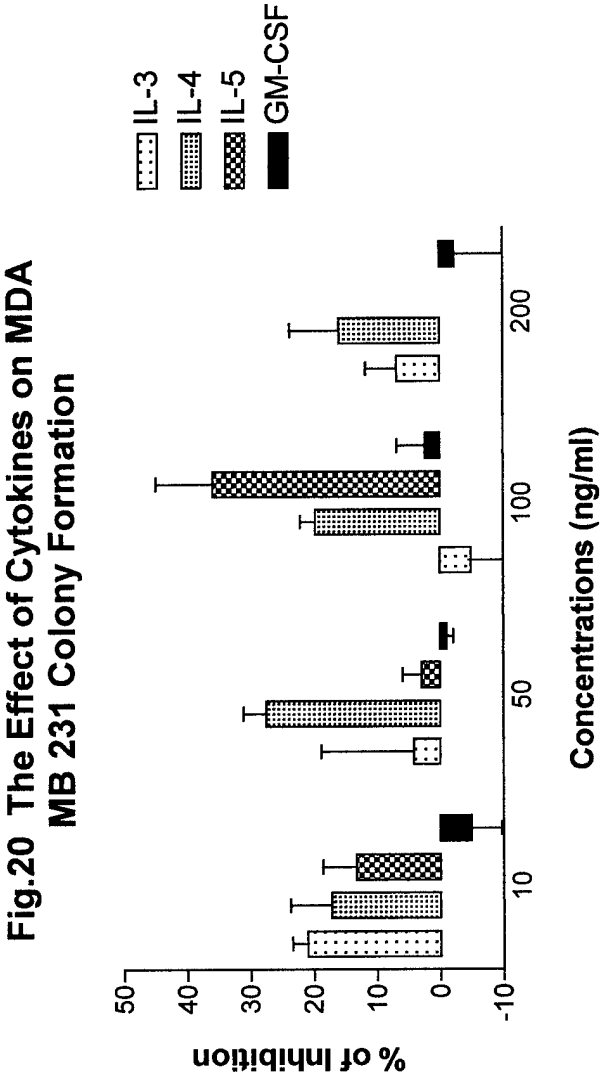
Fig.20 The Effect of Cytokines on MDA MB 231 Colony Formation
IL-3
IL-4
IL-5
GM-CSF
% of Inhibition
50
40
30
20
10
0
-10
10
50
100
200
Concentrations (ng/ml)

Fig. 20. MDA-MB-231 tumor cells were seeded; incubated with cytokines and examined for colony formation as described in fig. 18.

Fig.21 Effect of TNF-a on MCF-7 Colony Formation

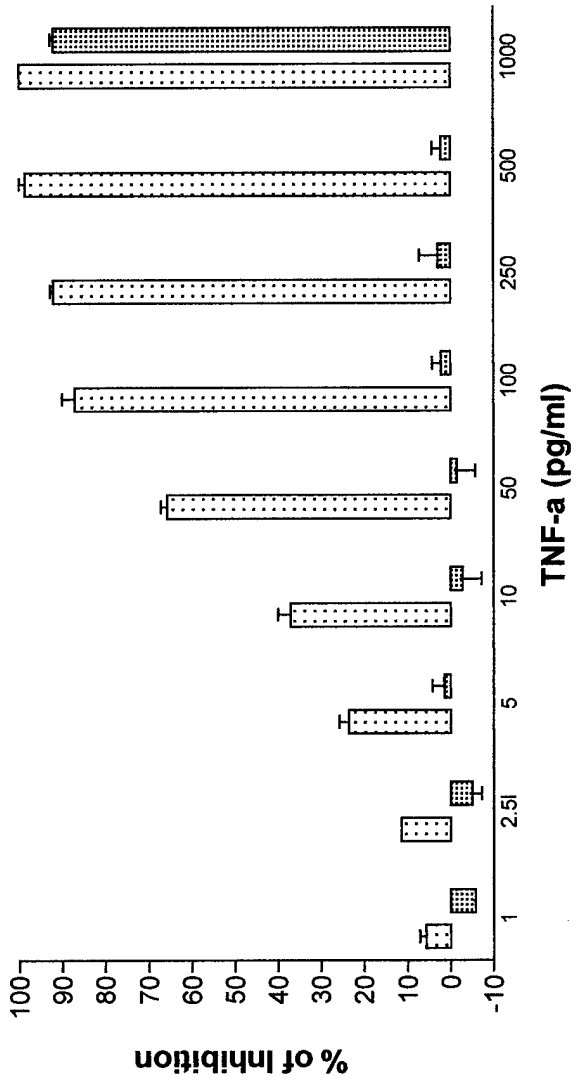

Fig. 21. MCF-7 tumor cells were seeded into wells of 6-well plate at 100 cells/well and treated with different concentrations of TNFα, (200ul/well. Each concentration and media control was set up in triplicate. Anti-TNFα (100ng/ml) was also added to certain wells. The plates were further incubated for 10 days.